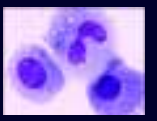
DIFFERENCES IN BAL MACROPHAGES CYTOLOGY DETERMINED BY MORPHOMETRIC ANALYSIS IN PATIENTS WITH SARCOIDOSIS



Smojver-Ježek S[1, 3], Peroš-Golubičić T[2,3], Vrabec-Branica B[1], Juroš Z[1].

[1]Department of Pathology and Cytology - Pulmonary Cytology Division, [2]Department of Lung Diseases, University Hospital Centre Zagreb, Croatia, [3]University of Zagreb, Medical School Zagreb, Croatia

BACKGROUND: Morphometric and image cytometric DNA analysis of the macrophages nuclei in BAL fluid of patients with sarcoidosis have been investigated in other to establish their diagnostic and clinical significance. In sarcoidosis macrophages are antigen presenting cells responsible for granuloma formation, but also secrete TNFa, IL-12, IL-15, IL-8 and different growth factors. Macrophages in sarcoidosis are larger than macrophages in healthy controls and posses different ultrastructural characteristics[1]. They are more uniform in appearance than in hypersensitivity pneumonitis and lack foamy characteristics[2]. Recently, three types of macrophages in sarcoidosis have been recognized ultrastructurally, 70% among them with signs of increased activity and cytokine release[3]. Sarcoidosis onset can be acute or chronic with different outcomes.

MATERIAL AND METHODS: Seventy-three patients were included in the investigation (Table 1). BAL fluid (BALF) was obtained during diagnostic flexible bronchoscopy. Inclusion criterion for all patients with interstitial lung disease was lymphocytic alveolitis. Morphometric and DNA image analysis were performed on macrophages nuclei in cytological specimens of BAL fluid. Archived originally MGG stained slides were restained with Feulgen method for morphometric and DNA image analysis of macrophages nuclei[4]. Random sampling was performed by systemic measurement of cells under microscope. Cells selection started at point inside a region with cellular material evenly distributed, proceeded cell by cell in one field, and continued to the next fields. Light microscopic analysis under oil immersion (x100) was applied and analysis was conducted in one focal nuclear plane. In each case 100 or more nuclei were analyzed, and processed with an image analyzer using SFORM software for digital image analysis (VAMSTEC, Zagreb). Objects contours were marked with special tools, interactive, by mouse selection (Figure 1). DNA image cytometric analysis of ploidy status was performed simultaneously as other morphometric measurements. Nuclei of neutrophilic granulocytes were used for internal controls on the same slide as investigated macrophages. DNA content was measured indirectly after quantitative DNA staining with Feulgen. IOD (integrated optical density) represents cytometric equivalent of DNA content, rescaling of the IOD values by comparison with IOD values of cells with known DNA content was necessary for quantification of nuclear DNA content of measured cell[5].(Figure 2). DNA image cytometry ploidy status was determined with Van Velthoven method. This method includes near-diploid hyperdiploid and triploid histogram types as aneuploid types. It is superior in describing changes in DNA content of non-malignant cells with high proliferative activity. DNA index (DI) has been cal-culated as ratio between modal value of the investigated cell peak divided by the modal value of the diploid refer-ence cell peak. Dyploid histograms had a DI value >0.90 <1.15, hyperdiploid >1.16<1.39, triploid >1.40<1.60, hypertriploid >1.61<1.89 and tetraploid >1.90<2.20[6]. Modification by Kardum-Skelin was used to calculate distribution of cells in histogram peak[7]. All investigated morphometric and DNA cytometric parameters are listed in Table 2.

Statistical Analysis

Statistical analysis was performed in Statistica for Windows version 6.0 (StatSoft, Inc. Tulsa, OK). Mean, median, standard deviation and minimum and maximum value have been calculated for all morphometric parameters. Mean, standard deviation and 95% confidence interval (95% CI) were used for description of variables and variance analysis was used for comparison between the groups. X^2-test was used for comparison of variables categories distribution among groups. Variables categories were represented as frequency (%). Classification criteria (diagnostic threshold values) were calculated with discriminant function analysis. Beckward step-wise method in multivariate discriminant function analysis was performed because of sample size and complexity.

GROUPS	DISEASES	No. of patients
1.	Sarcoidosis with acute onset (S1)	16
2.	Sarcoidosis with chronic onset (S2)	17
3.	Interstitial lung diseases other then sarcoidosis (NS)	30
4.	Controls (without lung disease) (C)	10
	Total	73

Table 1. Investigated groups of diseases and number of patients

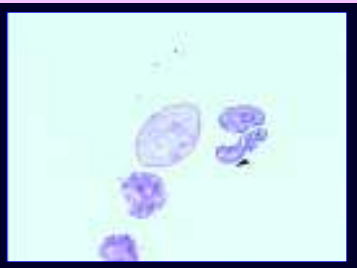


Figure 1. Marked contours of investigated (red) and control (blue) nuclei in morphomtric analysis procedure. Feulgen staining, original magnification x1000.

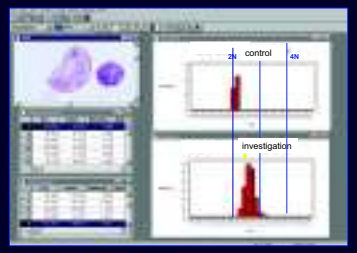


Figure 2. Tables and histograms with IOD values. Software for morphometry and DNA cytometry "Sform", Vamstec, Croatia

INVESTIGATED PARAMETERS		
MORPHOMETRIC (10)	SIMPLE Area Outline Convex area Length Bredth Maximal radius Minimal radius	COMPLEX Form factor (FF) Elongation factor (EF) Area /convex area (ACA)
DNA CYTOMETRIC (6)	DNA index (DI) Percentage of cels before histogram peak Percentage of cells in peak Percentage of cells after peak Percentage of cells in S-phase Percentage of cells in G2M phase	

Table 2. Investigated morphometric and DNA cytometric parameters

RESULTS:

R I.

Variables of mophometric parameters and statistical differences among groups of disease (variance analysis – ANOVA and Xi -test (p-values)

Statistical differences among groups p<0,001 - ↑↑, p<0,05 - ↑, non-significant - ns

Mean (Mn), median (Md), standard deviation (SD), minimum value (Min), maximum value (Max)

NO SINGLE VARIABLE OF INVESTIGATED MOPHOMETRIC PARIMETERS CAN DIFFERENTIATE GROUPS OF SARCOIDOSIS WITH ACUTE AND CHRONIC ONSET

MORPHOMETRY	Mn	Md	Max	Min	SD
Area	↑↑				
Outline	↑				
Convex area	↑↑				
Length	↑↑				
Bredth	ns		↑		
Maximal radius	↑				
Minimal radius	ns	↑	ns	↑	
FF	ns		↑		ns
EF	↑			ns	↑↑
ACA	↑	ns		↑	
DNA CYTOMETRY					
% before peak	ns				
% in peak	ns				
% after peak	ns				
% S-phase	↑				
% G2M phase	ns				
DI (X²-test)	↑				

R II.

Distribution of DI (DNA index) values of macrophages nuclei among groups and statistical differences (X^2 test)

X^2 = 10,229 df=3 p=0,017

	Hyperdiploid	Triploid/ hypertriploid	All
S1	6	10	16
S2	5	12	17
NS	5	25	30
C	7	3	10
All	23	50	73

S1- sarcoidosis with acute onset, S2 – sarcoidosis with chronic onset, NS – interstitial lung diseases other then sarcoidosis, C- contols (without lung disease)

R III.

Differences in % of macrophages nuclei in S-phase among investigated groups (multivariate discriminant function analysis, p-values)

	S1	S2	NS	C
S1		0,022	0,045	0,004
S2	0,022		0,550	0,355
NS	0,045	0,550		0,134
C	0,004	0,355	0,134	

S1- sarcoidosis with acute onset, S2 – sarcoidosis with chronic onset, NS – interstitial lung diseases other then sarcoidosis, C- contols (without lung disease)

R IV. MORPHOMETRIC AND CYTOMETRIC CHARACTERISTIC OF MACROPHAGES IN GROUPS OF INVESTIGATED DISEASES

SARCOIDOSIS WITH ACUTE ONSET	SARCOIDOSIS WITH CHRONIC ONSET	CONTROLS WITHOUT LUNG DISEASE	INTERSTITIAL LUNG DISEASES OTHER THEN SARCOIDOSIS
LARGEST UNIFORME	PROMAJNCED DIVERSITY	SMALL ROUND	LARGEST MORE INDENTED AND ELONGATED
INCREASED PROLIFERATION DECREASED APOPTOTIC ACTIVITY	INCREASED PROLIFERATION DECREASED APOPTOTIC ACTIVITY	SLIGHTLY INCREASED PROLIFERATION	INCREASED PROLIFERATION DECREASED APOPTOTIC ACTIVITY
↑↑ Mn and Md values of morphometric parameters ↓↓ EF ↑↑ % S-ph, G2M ph ↓↓ % in peak	↑↑ Mn, SD i max. values of morphometric parameters ↑↑ % before peak ↓↓ after peak ↑ = % S-ph	↓↓ Mn values of morphometric parameters ↓↓ % S-ph, G2M ↑↑ % in and after peak	↑↑ Mn and Md values of morphometric parameters ↓↓ % before peak ↑↑ % S-ph

Mn – mean, Md- median, SD- standard deviation, EF- elongation factor

R V. Multivariate discriminant step-wise analysis: 43 variables (yelow fields) of 15 morphometric and DNA cytometric parameters allowing 100% separation of sarcoidosis patients in groups with acute and chronic sarcoidosis

VARIABLES OF MORPHOMETRIC PARAMETERS	Mean	Median	Max values	Min values	SD
AREA					
OUTLINE					
CONVEX AREA					
LENGHT					
BREADTH					
MAXIMAL RADIUS					
MINIMAL RADIUS					
FORM FACTOR					
ELONGATION FACTOR					
ACA (AREA/CONVEX AREA)					

VARIABLES OF DNA CYTOMETRIC PARAMETERS	% before peak	% in peak	% after peak	% S-phase	% G2M phase

R VI. Classification functions of parameters variables with coefficients and constants for each investigated group of diseases (multivariate discriminative analysis - *backward step-wise*). *Part of the original record.*

Classification functions; grouping				
	K	S1	S2	N
EF_Mn	3911145	3911066	3912739	3913008
EF_Md	-25454	-25549	-25383	-25456
LOG_EF_min	1154324	1154239	1154888	1154515
FF_Mn	2161281	2161203	2162013	2162487
FF_Md	-2146834	-2146873	-2147454	-2147862
LOG_FF_SD	-38934	-39032	-38954	-38992
Constant	-210696509	-210706498	-210754444	-210758215

R VII.

CORRECT CLASSIFICATION OF BAL MACROPHAGES NUCLEI POPULATIONS IN GROUP OF DISEASES

Calculation:

Value of variable1 x coefficient + value of variable2 x coefficient + ...+ .... + value of variable 43 x coefficient + constant = score

Score have to be calculated for every group

Score that is largest among groups allows classification of macrophages population in that particular group of diseases and

CORRECT CLASSIFICATION OF SARCOIDOSIS WITH ACUTE OR CHRONIC ONSET

Final result of investigation is

MATHEMATICAL MODEL

COMBINATION OF MEASURED VALUES AND COEFFICIENTS AND CONSTANTS of 43 VARIABLES after morphometric and statistical analysis of

15 morphometric and DNA cytometric parameters of 10706 macrophages nuclei in BALs of 73 patients

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